REMARKS

Claims 11-15, 21 and 22 are pending. No claim has been allowed.

Rejection Under 35 U.S.C. §§ 101 and 112, first paragraph

Claims 11-15, 21 and 22 remain rejected under 35 U.S.C. §§ 101 and 112, first paragraph for reasons of record. More specifically, in the Advisory Action, the Examiner acknowledges that the RANKL "may be associated with inflammation somehow" but finds the evidence presented to date lacking because "there is no information on how [RANKL] is involved." According to the Examiner, it is not the accuracy of the quantitation that the specification should disclose but the how the RANKL protein is functioning. Applicants traverse this rejection.

A. The appropriate standard for the utility requirement is not being applied

In rejecting the presently claimed invention, the Office has apparently taken the position that only certain evidence substantiated by actual experimental data establishes patentable utilities. However, such is not the legal standard for the utility requirement. If an invention has at least one stated objective use, then utility under 35 U.S.C. § 101 is clearly shown. MPEP § 2107.02 (I) (8th Ed. (Latest Rev.) 2004). In other words, the disclosure should convey to the person of skill in the art to readily understand why the invention is useful.. *Id.* at § 2107.02 (II). Section 101 simply requires that the asserted utility be specific, substantial, and credible. There is no legal requirement that the disclosed utility must be supported by conclusive experimental data. The sufficiency of the disclosure is based on "whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of the evidence and reasoning provided." M.P.E.P. § 2107.02 (III) (B).

Appellants respectfully submit that the Office appears to have adopted an incorrect standard in maintaining the instant rejection. Specifically, the Office appears to be requiring a certain and exact evidence be disclosed for RANKL if the specification is to meet the utility requirement of §§ 101 and 112, *i.e.*, that the specification precisely state the biological role of RANKL in inflammation. In essence, the Office is requiring proof beyond a reasonable doubt regarding the role of RANKL in inflammatory responses.

B The specification discloses specific utilities for RANKL

The specification provides at least two specific, substantial, and credible utilities for RANKL sufficient to satisfy 35 U.S.C. § 101. First, the specification discloses a specific utility for RANKL as useful in the modulation of immune responses such as inflammation. As discussed in previous responses of record, the specification discloses that "[RANKL] plays a role in regulation or development of hematopoietic cells ... lymphoid cells, which affect immunological responses" in view of RANKL's homology to other known TNF receptors. *See* specification, page 57, lines 22-24. Second, based on the disclosed homology to the TNF receptor family, a person of skill in the art would recognize and find this asserted utility credible for RANKL as it is well known in the art that TNF receptor family members play a crucial role in the development and regulation of immune and inflammatory responses, often through the induction of apoptosis. *See, e.g.*, Exhibit A at page 3. Finally, the specification provides objective evidence to support this evidence. As discussed in previous responses of record, Applicant discloses that RANKL is highly expressed in allergic lung with little expression in normal lung in two different animal models for inflammation/allergy models, providing objective evidence of the asserted utility.

The specification also discloses another specific utility for RANKL - a regulator of cell proliferation or development. *See* the specification at page 32, lines 19-21. In particular, the specification cites the ability to kill cells (*e.g.*, induce apoptosis), affect differentiation, and cause changes in cytokine expression as specific examples of regulating cellular proliferation. *See* the specification at page 32, lines 22-32. Therefore, Applicants disclose that RANKL antibodies may be useful in the treatment of diseases associated with abnormal proliferation including cancerous conditions and degenerative conditions. *See* the specification at page 57, lines 8-25.

Both of the disclosed utilities for RANKL are specific, substantial, and credible. First, the disclosed utilities are sufficiently specific. While each of the disclosed utilities is broad, this does not render the utility non-specific or generic to any new protein. For example, the specification describes detailed applications for RANKL in a modulation of the immune response and how this may be useful therapeutically, even suggesting various compositions that would be useful with RANKL. *See e.g.*, the specification at page 57, line 26 to page 58, line 28. Likewise, Applicants also disclose RANKL as useful in the treatment of degenerative diseases or cancerous

conditions as one application of its anti-proliferation activity. *See, e.g.*, the specification at page 57, lines 9-16. These particularized utilities for RANKL constitute real world uses for the novel protein. It is clear from the disclosure that RANKL (and antibodies specific for RANKL) can be used for modulation of immune responses and other diseases associated with proliferation disorders, particularly in view of their homology to other TNF receptor family members. A person of skill in the art would readily recognize and believe that a protein involved in apoptosis can play a critical role both in degenerative diseases and cancerous conditions. Thus, a hunting license is not required to determine a use for RANKL as it is an identified cytokine in a well known family with data that supports the asserted utilities. Finally, the asserted utilities are credible to a person of ordinary skill. The roles for TNF receptors in modulation of cellular proliferation, particularly in the induction of apoptosis, and in the immune response are well recognized and non-controversial. *See, e.g.*, Exhibit A at page 3. Thus, the asserted utilities meet the standard of 35 U.S.C. § 101.

Applicants respectfully submit that there is no legal requirement that mandates that the exact or precise biological activity of RANKL be identified. It is insufficient to merely state that a protein or compound has "biologic activity" without more. However, that is not the case here. The specification discloses specific utilities. While these are broad categories, *e.g.*, modulation of cellular proliferation, it is well know that TNF receptors are able to module the proliferation of a number of different cell types, primarily through the induction of apoptosis. This potent and broad activity does not correspond to a generalized biological activity. While broad, the activity of RANKL is sufficiently identified and bolstered with objective evidence to meet the legal requirements set out in the statute. Nothing more is required of Applicants.

C. Overwhelming objective evidence supports the asserted utilities

Applicants respectfully submit the evidence disclosed in the specification and that made of record provide ample support for the disclosed utility for RANKL in modulating immune responses. The specification discloses data examining mRNA expression of RANKL in a number of systems. RNA expression was observed in fetal tissues and in a number of art-recognized inflammatory and allergic animal models. Subsequently, Applicants provided the declaration of Jeanine Mattson attesting to and providing additional objective evidence using a very sensitive detection technique known as Taqman. *See* Declaration of Jeanine D. Mattson Pursuant to 37

C.F.R. § 1.132 submitted on April 28, 2003. This data demonstrates that RANKL was upregulated in art-recognized models of inflammation and allergy, thus indicating a role for RANKL in inflammation and allergy, *i.e.*, development and regulation of immune responses. Applicants also provided a peer-reviewed article demonstrating that a person of skill in the art relied on Taqman data to assert a utility for a cytokine in a particular disease. In sum, Applicants provided specific, supporting evidence of function in art-recognized disease models using technology routinely employed by skilled artisans to discern a role for a cytokine in a specific disease.

Objective evidence also unequivocally supports the asserted utility of RANKL as a modulator of cellular proliferation. Applicants provided herewith one example of RANKL acting to modulate cellular proliferation. *See* Exhibit A. Briefly, the authors of this article demonstrate that RANKL triggers apoptosis and thereby limits cellular proliferation. It mediates apoptosis using the FADD/caspase 8 pathway commonly used by members of the TNF receptor family. Similar to Applicants, the authors of the article assert utilities for RANKL in view of its ability to modulate proliferation that include a role in development and cancer treatments. *See* Exhibit A at page 18 and the specification at page 57, lines 8-16. Thus, the utility of RANKL in modulating cellular proliferation, particularly through the elicitation of apoptosis, is supported by objective evidence.

The evidence of record sufficiently supports the asserted utilities, when considered as a whole, because it leads a person of skill in the art to conclude the asserted utility is more likely than not true. MPEP § 2107.02 (VII). Applicants note that the Examiner has been unpersuaded by an overwhelming amount of data that would be sufficient for a person of skill in the art. For example, Applicants provided the declaration of an expert in the field of mRNA expression analysis and additional data by the Taqman detection technique following the Examiner's refusal to give any weight to the mRNA expression disclosed in the specification. The data provided in the declaration fully corroborated the data presented in the specification. Applicants also provided a peer-reviewed article using Taqman mRNA expression to examine an inflammatory model to provide additional support for the Taqman method are sufficient in the art to provide the asserted basis for a utility in a specific disease. The Examiner initially argued that the mRNA expression data in the specification and in the Taqman data was unpersuasive because the mRNA expression data did not necessarily correlate with protein expression. In the recent Advisory Action, the Examiner acknowledges that

the transcript levels are adequately expressed such that protein expression can be implied, but continues to dismiss the data asserting that Applicants are required to provide information about how RANKL is involved in inflammation. There is no requirement for providing a mechanism for a protein or compound in order to meet the utility requirement. Moreover, contrary to the Examiner's assertion, Li is not distinguished from the instant invention by the fact that IL-1β is known to be an inflammatory cytokine. Li provides no suggestion that the inflammatory character of IL-1β formed the basis of his conclusion. He looked merely to cytokine expression. Likewise, Applicants rely on the Taqman data (and corroborating mRNA expression data) to assert that RANKL has a role in inflammation and allergy. As this is a specific, substantial, and credible utility supported by a totality of the evidence sufficient to convince a person of skill in the art, nothing more is required.

In view of the above, Applicants submit that the basis of the rejection may be removed.

Rejection Under 35 U.S.C. § 102 (e)

Claims 11-14, 21 and 22 are rejected under 35 U.S.C. § 102 (e) as allegedly being anticipated by Goddard et al., U.S. Published Application 20030092044, effective filing date April 12, 1999 for reasons of record. Applicants traverse this rejection.

Applicants submit that Goddard is not a proper reference under 35 U.S.C. § 102 (e) for reasons of record. Again, Applicants have met their burden with regards to the utility requirement for both the protein and the nucleic acid of the RANK-like protein, and therefore, Applicants maintain their traversal of the Examiner's refusal to properly award a priority date of at least September 11, 1998. As the priority date is properly at least September 11, 1998, Goddard is not a reference under 35 U.S.C. § 102 (e) available against the instant application.

In view of the above, Applicants submit that the basis of the rejection may be removed.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 140942000401. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Respectfully submitted,

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Induction of apoptosis by X-linked Ectodermal Dysplasia Receptor via a Caspase 8-dependent mechanism

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EXHIBIT A

Abstract

XEDAR[1] is a recently isolated member of the Tumor Necrosis Factor Receptor (TNFR) family that is highly expressed during embryonic development and binds to ectodysplasin-A2 (EDA-A2). In this report, we demonstrate that although XEDAR lacks a death domain, it nevertheless induces apoptosis in an EDA-A2-dependent fashion. The apoptosis-inducing ability of XEDAR is dependent on the activation of caspase 8 and can be blocked by its genetic and pharmacological inhibitors. Although XEDAR-induced apoptosis can be blocked by dominant-negative FADD and FADD siRNA, XEDAR does not directly bind to FADD, TRADD or RIP1. Instead, XEDAR signaling leads to the formation of a secondary complex containing FADD, caspase 8 and caspase 10, which results in caspase activation. Thus, XEDAR belongs to a novel class of death receptors that lack a discernible death domain but are capable of activating apoptosis in caspase 8 and FADD-dependent fashion. XEDAR may represent an early stage in the evolution of death receptors prior to the emergence of death domain and may play a role in the induction of apoptosis during embryonic development and adult life.

[1] The abbreviations used are: XEDAR, X-linked Ectodermal Dysplasia Receptor; TNF, Tumor Necrosis Factor; TRAIL, TNF-related apoptosis-inducing ligand; TNFR1, TNF Receptor 1; FADD, Fas-associated death domain, TRADD, TNF Receptor associated death domain; siRNA, small interfering RNA, DD; death domain; DR4; Death receptor 4; DR5, Death receptor 5;; EDAR; Ectodermal Dysplasia Receptor; EDA, Ectodysplasin A; cFLIP, cellular FLICE inhibitory protein.

Introduction

The death receptors of the Tumor Necrosis Factor Receptor (TNFR) superfamily and their ligands have been recognized to play a crucial role in the normal development and regulation of immune and inflammatory response (1,2). The apoptosis inducing ability of these receptors has been mapped to a conserved cytoplasmic domain of 60-80 amino acids, called the death domain (DD) (1,3). TNFR1 is the prototypical and perhaps the best characterized death receptor (4). Recent studies suggest that ligand-induced trimerization of TNFR1 leads to the recruitment of DD-containing adaptor protein TRADD to a plasma membrane bound complex (complex I) (5-7). TRADD helps in the recruitment of DD-containing serine/threonine kinase RIP1 and adaptor protein TRAF2 (5-7). Assembly of Complex I occurs in lipid rafts and leads to NF-κB activation via RIP1-mediated recruitment of the IKK complex whereas JNK is activated via TRAF2-mediated activation of MAP3-kinase (5,6). Subsequently, TRADD and RIP1 dissociate from complex I and associate with a cytoplasmic complex (complex II) consisting of DD-containing protein FADD and procaspase 8, the apical caspase of the caspase cascade (5). Under conditions favoring TNFR1-induced apoptosis, procaspase 8 is activated upon recruitment to complex II and subsequently results in the activation of downstream caspases, such as caspase 3, 6 and 7, and eventual cell death (5). Unlike TNFR1, signaling via Fas, DR4 and DR5 delivers a strong and rapid pro-apoptotic signal (3,8,9). Ligand binding to these receptors leads to DD-mediated recruitment of FADD directly without the involvement of TRADD (8-10). FADD subsequently leads to the recruitment and activation of procaspase-8 (3,8).

Ectodysplasin A is a distantly related ligand of the TNF family that plays a key role in ectodermal differentiation (11). Mutations in the ectodysplasin gene *(Eda)* causes X-linked hypohidrotic ectodermal dysplasia, which is characterized by the absence or deficient function of hair, teeth and sweat glands (12,13). Several alternatively spliced transcripts of EDA have been identified (14-16). The two predominant splice variants, EDA-A1 and EDA-A2, differ from each other by a two amino acid motif and bind to distinct receptors (17). Thus, EDA-A1 binds to a TNF family receptor designated EDAR, while EDA-A2 binds to the related receptor, XEDAR (17). Transgenic expression of a secreted form of EDA-A2 resulted in thin and listless animals which died within 1 month of birth (18). Histological examination of EDA-A2 transgenic animals exhibited multifocal myodegeneration (18). The downstream events culminating in EDA-A2-induced myodegeneration and early lethality are unclear at present.

Unlike most TNFR family receptors, XEDAR is a type III transmembrane protein (lacking an NH2-terminal signal peptide) which bears 32% sequence homology with EDAR in the extracellular ligand-binding domain (17). However, XEDAR possesses a unique intracellular region with no significant homology to other TNFRs. Two predominant alternatively spliced isoforms of XEDAR have been described (XEDAR-s and XEDAR-L) which differ from each other by the presence of a 21 amino acids linker in the juxta-membrane region of the cytoplasmic domain.(19). Both XEDAR isoforms lack a death domain and have been shown to signal mainly via TRAF6 and TRAF3 to activate the NF-κB and JNK pathways (17,19).

In this study, we report that although XEDAR lacks a death domain, it nevertheless possesses the ability to induce programmed cell death. Unlike Fas and the TRAIL receptors, XEDAR does not directly bind to the death adaptors FADD, TRADD or RIP but activates caspase 8 via the formation of a complex containing FADD, caspase 8 and caspase 10. Our results suggest that XEDAR may represent an early stage in the evolutionary history of death receptors and may play a role in the mediation of apoptosis during development and in adult life.

Materials and Methods:

Cell Lines and Reagents:

293Flag-XEDAR cells and expression plasmids encoding Flag-XEDAR, its C-terminal deletion mutant N172, crmA, Flag-DR4, Flag-TNFRI, caspase 8 C360S and $cFLIP_L$/ MRITα-1 have been described previously (19,20). Rabbit polyclonal antibodies against RIP, TRAF1, TRAF3, TRAF6, β-actin, and goat polyclonal against FADD, RICK, Caspase8 and TRADD were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Caspase 8, cleaved caspase 3, Caspase 9, cleaved PARP and BID were obtained from Cell Signaling (Beverly, MA). FLAG and control mouse IgG beads and mouse monoclonal anti-FLAG (M2)-HRP, Tubulin were obtained from Sigma (St. Louis, MO). Caspase assay substrates DEVD-AFC, IETD-AFC and LEHD-AFC and cell permeable caspase inhibitors and were purchased from Enzyme Systems and Calbiochem (La Jolla, CA). Recombinant human EDA-A2 and TNF_ were obtained from R &D systems and also generated in SF9 cells as described earlier (19). XEDAR-Fc and EDAR-Fc were generated as described previously (19,20). TNFR1-Fc and a neutralizing antibody against human TNFα were obtained from Peprotech Inc. (Rocky Hill, NJ).

Co-immunoprecipitation Assays:

For studying *in vivo* interactions, 5 X 10^7 293F-XEDAR-L cells were treated with control supernatant or EDA-A2 for ten minutes. Cells were subsequently lysed in 5 ml of buffer A (20 mM sodium phosphate (pH 7.4), 150mM NaCl) containing 5% glycerol, 1% Triton-X 100, and 1 EDTA-free mini-protease inhibitor tablet per 10 ml (Roche). Cell lysates were precleared with mouse IgG beads and then incubated for 1 hour at 4°C with 50 µl of FLAG beads precoated with a super-saturated casein solution. Beads were washed twice with buffer A, once with a high salt wash buffer (buffer A + 500 mM NaCl) and again with buffer A. Bound proteins were eluted by boiling, separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot. Co-immunoprecipitation of caspase 8 complex was performed using 2µg of an anti-caspase 8 antibody (Santa Cruz) and essentially as described previously (5).

Caspase Activation Assay:

293F-XEDAR-L cells were left untreated (control) or treated with EDA-A2 or TRAIL (50 ng/ml) for different time intervals and subsequently lysed in buffer C (50 mM HEPES, pH 7.4, 1 mM EDTA, 10 mM, 10 mM DTT, 10% glycerol and 0.1% CHAPS). Caspase activity was measured in 100 µl reaction volume containing cell lysate (20 µg of total protein) and 5 µM of AFC-coupled peptide substrates in buffer D (buffer C containing 100 mM NaCl). The reaction was followed at 37°C by SpectraFluor using 400 nm excitation and 510 nm emission filters. Caspase activities were estimated by

measuring the turnover of substrates DEVD-AFC (caspase 3), IETD-AFC (caspase 8), and LEHD-AFC (caspase 9) (Enzyme systems).

Apoptosis Assays:

Cells (1.5-3 x 10^5) were transfected with the empty vector, XEDAR, TNFRI or Fas (500 ng) along with β-lactosidase- and GFP-encoding plasmids (75 ng each) using calcium phosphate (293T and 293F) or lipofectamine (L-929 cells). Cells were examined under a fluorescent microscope and photographed 36 h after transfection, or were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, as described previously (21). β-galactosidase positive cell were counted for viable and apoptotic cells based on their morphology. For all ligand induced cell death experiments, stable cells expressing XEDAR were treated with EDA-A2 or TRAIL for 12 hours and then stained with Hoechst 33342 and/or YOPRO-1. Cells were visualized by phase contrast and fluorescence microscopy. Approximately 300 cells were counted for each treatment (n=3) from three randomly selected fields and mean was used to calculate percentage apoptosis. Each experiment was repeated at least three times. Cell permeable synthetic caspase inhibitors Boc-D-fmk, zVAD-fmk, zDEVD-fmk, zVDVAD-fmk, zVEID-fmk, zIETD-fmk, and zLEHD-fmk, were dissolved in DMSO and added to the cells just before treatment with the ligand. DMSO was added to the control wells to the same final concentration. For experiments testing the involvement of TNFα/TNFR1 in EDA-A2 induced apoptosis, TNFα, TNFR1-Fc and a neutralizing mAb against TNFα were used at concentrations of 10ng/ml, 0.2μg/ml and 0.5μg/ml, respectively.

Preparation and Transfection of siRNA

siRNA oliginucleotides with two thymidine residues (dTdT) at the 3'-end of the sequence were designed against caspase 8 (sense, 5' -GCAAGAACCCAUCAAGGAUdTdT- 3'), FADD-1 (sense, 5'-CGUCAUAUGUGAUAAUGUGdTdT -3'), FADD-2 (sense, 5'-CCGAGCUCAAGUUCCUAUGdTdT-3'), TRADD (sense, 5' – AACUCCACUUGGCCUAUCUdTdT-3'), mouse TRADD (control) (sense, 5'-UAUACAAGGCUCUGCAGACdTdT-3') and Lamin A/C (sense, 5' CUGGACUUCCAGAAGAACAdTdT -3') along with their corresponding antisense oligonucleotides. 293Flag-XEDAR cells were transfected with 80 nM of ds RNA using calcium phosphate in each well of a 6 well plates and re-plated into wells of a 24 well plate 24 hr post-transfection. 48 to 60 hours post transfection cells were treated with EDA-A2 or TRAIL (50 ng/ml) for another 12 hours. An untreated replicate from each set was analyzed by Western blotting to confirm the down regulation of the protein targeted by siRNAs.

Results

Recombinant EDA-A2 induces apoptosis in 293F cells expressing XEDAR

In order to facilitate the characterization of XEDAR signaling, we recently generated a subclone of 293F (human embryonic kidney), designated 293Flag-XEDAR, with stable expression of N-terminal Flag-epitope tagged XEDAR-L isoform (19). These cells were generated using retroviral-mediated gene-transfer and show modest expression of XEDAR as determined by cell-surface staining with an antibody against the Flag tag. While studying the ability of EDA-A2 to induce NF-κB pathway in these cells, we were surprised to find that a large number of cells were undergoing cell death (Fig. 1A). EDA-A2 treated cells demonstrated typical features of apoptosis, such as cell rounding,

detachment and fragmentation into small apoptotic bodies (Fig 1A, top). They also readily stained with YOPRO-1, a cell impermeable nuclear dye that stains only those cells that have lost membrane integrity (22) (Fig 1A, middle). Furthermore, nuclear staining with YOPRO-1 and Hoechst 33342 dyes showed that the nuclei of EDA-A2-treated cells were condensed and fragmented, another characteristic of apoptotic cells (Fig 1A, inset). We confirmed the apoptosis-inducing ability of EDA-A2 using several independent preparations of this protein prepared in our laboratory as well as a commercially available preparation (R&D Systems) (not shown). The cytotoxicity of EDA-A2 was specific for XEDAR-expressing cells as it had no effect on parental 293 cells or those infected with a control retroviral vector (not shown). Furthermore, EDA-A2-induced cell death could be specifically blocked by soluble XEDAR (XEDAR-Fc), whereas soluble EDAR (EDAR-Fc) was without any inhibitory effect (Fig. 1C). EDA-A2 also induced apoptosis in a stable clone of HeLa cells expressing XEDAR, although these cells required sensitization with actinomycin-D (Fig. 1B).

It has been reported that the cytotoxic effects of TNFR2, CD40 and CD30 are mediated by the endogenous production of TNFα, which activates TNFR1 in an autotropic or paratropic fashion (23). Therefore, we sought to determine the role of TNFα/TNFR1 signaling in the induction of apoptosis by EDA-A2. Unlike EDA-A2, treatment of 293Flag-XEDAR cells with TNFα (in the absence of actinomycin-D) failed to induce significant apoptosis, thereby arguing against the possibility that EDA-A2 induces apoptosis via the production of endogenous TNFα (Fig 1D). More importantly, blockage of TNFα/TNFR1 signaling by the use of soluble TNFR1 receptor (TNFR1-Fc) or a

neutralizing antibody against TNFα had no significant effect on apoptosis induced by EDA-A2, while successfully blocking apoptosis induced by combined treatment with TNFα and actinomycin-D (Fig 1E and F). Taken together, the above results demonstrate that EDA-A2 induced apoptosis is not mediated via the production of TNFα and resultant signaling viaTNFR1.

Induction of apoptosis by XEDAR

Unlike the classical death receptors, XEDAR does not possess a death domain and, therefore, the ability of EDA-A2 to induce apoptosis in XEDAR-expressing cells was unexpected. Signaling via the receptors of the TNFR family can be activated in a ligand-independent fashion by overexpression-induced receptor aggregation (19). Therefore, we sought to determine whether over-expression of XEDAR, by itself, would induce apoptosis. As shown in Fig. 2A and C transient transfection of plasmids encoding full-length XEDAR-L or XEDAR-s isoforms (19), but not a C-terminal deletion mutant (N172), in 293T cells led to cellular rounding, condensation detachment and fragmentation into apoptotic bodies, features suggestive of cell death. We obtained essentially similar results upon transient transfection of XEDAR in mouse L-929 cells (Fig. 2D).

Activation of extrinsic caspase pathway during EDA-A2-induced apoptosis

The presence of nuclear fragmentation during EDA-A2-induced apoptosis pointed towards the involvement of caspases. Therefore, in order to test whether caspase activation is involved in XEDAR induced apoptosis, we treated 293Flag-XEDAR cells

with EDA-A2 and TRAIL (positive control) and analyzed the cell lysates for cleavage of various caspases known to be involved in the induction of apoptosis. We readily detected cleavage of caspase 8 into p43, p41 and p18 fragments within 8 hours of EDA-A2 treatment, although weak cleavage was evident within 4 hours (Fig. 3A). Caspase 3 is one of the executioner caspases of the caspase cascade and is activated by caspase 8 during apoptosis induced by the death receptors. We detected significant cleavage of caspase 3 into its active fragments, p19 and p17, following EDA-A2 treatment (Fig 3A). EDA-A2 treatment also led to the cleavage of PARP, one of the caspase 3 substrates, indicating caspase 3 activation (Fig. 3A). We confirmed activation of caspase 8 and 3 in the lysates of EDA-A2 treated cells using their synthetic peptide substrates coupled with a fluorogen AFC (Fig. 3B, C).

Activation of intrinsic (mitochondrial) caspase pathway during EDA-A2-induced apoptosis

Bid is a pro-apoptotic member of the bcl2 family that is cleaved by caspase 8 during apoptosis induced by death domain-containing receptors of the TNFR family (24,25). Truncated Bid (tBid) then translocates to the mitochondria, where it induces release of cytochrome *c* into cytosol and subsequent activation of caspase 9. As shown in Fig. 4A, a cleaved fragment of BID (p15) appeared as early as 4 hours after EDA-A2 treatment and correlated with the appearance of the cleaved fragments (p37/35) of caspase 9. Activation of caspase 9 following EDA-A2 treatment was further confirmed using a fluorogenic assay based on the cleavage of its synthetic peptide substrate (Fig. 4B). Treatment with EDA-A2 also resulted in the release of cytochrome c and Smac from the mitiochondria

into the cytosol (Fig 4C). TRAIL was used as a positive control for the above experiments. Taken together, the above results demonstrate activation of both intrinsic and extrinsic cell pathways of caspase activation following EDA-A2 treatment.

Caspase activation is required for EDA-A2-induced apoptosis

We next sought to determine whether caspases are functionally involved in EDA-A2 induced apoptosis. As shown in Fig 5A, Boc-D.fmk and zVAD-fmk, two broad-spectrum synthetic cell-permeable caspase inhibitors, effectively blocked EDA-A2-induced cell death. zIETD-fmk, a preferential inhibitor of caspase 8, was similarly very effective in blocking EDA-A2 induced apoptosis (Fig 5A). In contrast, preferential inhibitors of caspase 3 (zDEVD-fmk) and caspase 9 (Z-LEHD-fmk) had only a partial inhibitory effect, while preferential inhibitors of caspase 2 (zVDVAD-fmk) and caspase 6 (zVEID-fmk) failed to significantly block EDA-A2-induced apoptosis (Fig. 5A). Finally, crmA, a cowpox virus-encoded preferential inhibitor of caspase 8 (26), also effectively blocked EDA-A2 induced apoptosis (Fig. 5B).

Caspase 8 is essential for EDA-A2-induced apoptosis

We further analyzed the contribution of caspase 8 to XEDAR-induced apoptosis by using its dominant-negative mutant (Caspase8 C360S) and cFLIP$_L$/MRITα1, a naturally occurring cytoplasmic inhibitor of caspase 8 (27,28). As shown in Fig. 5C, both caspase 8 C360S mutant and cFLIP$_L$/MRITα1 blocked XEDAR-induced apoptosis with efficiency comparable to that against Fas, a receptor known to use caspase 8 for induction of cell death. EDA-A2-induced apoptosis was also effectively blocked by siRNA-mediated down-regulation of caspase 8 expression. In fact, caspase 8 siRNA blocked cell death

induced by EDA-A2 with efficiency comparable to that against TRAIL, a TNF family ligand known to require caspase 8 for the induction of apoptosis (Fig. 5D-F). Interestingly, siRNA-mediated downregulation of caspase 8 expression led to complete inhibition of caspase 3 activation, thereby demonstrating that activation of caspase 8 is required for activation of caspase 3 following EDA-A2 treatment (Fig. 5G, H). Taken together, the above results establish caspase 8 as the apical caspase in EDA-A2/XEDAR induced apoptosis and demonstrate that it plays a non-redundant and indispensable role in this process.

Role of FADD in EDA-A2-induced apoptosis

FADD is a common mediator of cell death induced by the classical death domain-containing receptors of the TNFR family and is recruited to them either directly or via the intermediate adaptor protein TRADD (3,8). We checked the involvement of FADD in XEDAR-induced cell death by using a dominant-negative mutant of FADD (DN-FADD) that lacks its death effector domain (29). We found that overexpression of DN-FADD blocked XEDAR-induced cell death in a dose-dependent fashion (Fig 6A). However, dominant-negative FADD was slightly less effective in blocking cell death induced by EDA-A2 as compared to that induced by TRAIL (Fig 6A). We used two different siRNAs directed against FADD to confirm its involvement in EDA-A2-induced cell death. As shown in Fig. 6B and C, we observed significant inhibition of apoptosis upon EDA-A2 treatment in cells in which FADD expression had been silenced using either of the two siRNAs. In contrast a siRNA directed against Lamin A/C failed to significantly block EDA-A2 induced apoptosis. However, consistent with our previous results using

DN-FADD, we observed that siRNAs against FADD were slightly less effective in blocking EDA-A2-induced apoptosis as compared to TRAIL-induced apoptosis (Fig 6B). Collectively, the above results suggest that while FADD is involved in EDA-A2-induced apoptosis it might play a role distinct from its role in TRAIL-induced apoptosis.

TRADD is a component of both complex I and II during TNFR1 signaling (5). Although, TRADD is generally believed to be a key mediator of FADD recruitment during TNFR1 signaling, genetic evidence of its involvement in TNFR1-induced apoptosis is lacking so far. We tested the involvement of TRADD in EDA-A2 and TNFα-induced apoptosis using the siRNA approach. Remarkably, siRNA-mediated silencing of TRADD expression failed to significantly block EDA-A2 or TNFα-induced apoptosis in 293FLAG-XEDAR cells while silencing of caspase 8 effectively did so (Fig 6D, E). These results argue against the involvement of TRADD in EDA-A2-induced apoptosis and suggest that its role in TNFR1-induced apoptosis may need re-examination.

Lack of recruitment of FADD, TRADD, RIP and Caspase 8 to XEDAR

We used a co-immunoprecipitation assay to test the involvement of FADD and TRADD in apoptosis-induction by XEDAR. For this purpose, 293FLAG-XEDAR cells were treated with EDA-A2 for 10 min or left untreated, following which cells were lysed and XEDAR immunoprecipitated with Flag antibody beads and presence of various endogenously-expressed interacting proteins in the immunoprecipitated samples detected by Western blot analysis. Consistent with our previously published results (19), we readily detected an interaction between stably expressed Flag-XEDAR and endogenous

TRAF6 and TRAF3 upon treatment with EDA-A2 (Fig. 7A). However, under similar conditions, XEDAR failed to recruit endogenous FADD or TRADD (Fig. 7A). Similarly, we did not detect an interaction between XEDAR and protein kinases RIP-1 or RICK/RIP2 (Fig. 7A). In an independent experiment, we transiently overexpressed Flag-XEDAR in 293T cells and tested its interaction with endogenously expressed adaptor proteins and caspase 8. Again, we readily observed an interaction between overexpressed XEDAR and endogenously expressed TRAF6 but failed to see an interaction between overexpressed XEDAR and FADD, TRADD or caspase 8 (Fig 7B). We used overexpression of DR4 as a positive control in this experiment and, as expected, readily detected its interaction with endogenously expressed FADD and caspase 8 (Fig 7B). Collectively the above results demonstrate that FADD, TRADD, RIP1 and RIP2 are not directly recruited to XEDAR during induction of cell death.

Evidence for a caspase-8, -10 and FADD-containing secondary complex during EDA-A2-induced apoptosis

Lack of recruitment of FADD and caspase 8 to XEDAR was reminiscent of signaling via TNFR1, in which caspase 8 and FADD form a secondary proapoptotic cytosolic complex (complex II) distinct from the receptor-containing membrane-associated complex (complex I) (5,7). As such, we sought to determine whether signaling via EDA-A2 will similarly lead to the formation of a secondary complex between caspase 8 and FADD. For this purpose, we immunoprecipitated caspase 8 from the lysates of 293FLAG-XEDAR cells with and without treatment with EDA-A2 for different time intervals. No association between caspase 8 and FADD was found in the untreated cells (Fig 7C).

Association started 4 hr post-stimulation and increased further at the 8 and 16 hr time points (Fig 7C). Interestingly, recruitment of FADD to caspase 8 complex corresponded to the time of its cleavage (Fig 7C). We also detected cleavage fragments of caspase 10 in the caspase 8 complex at the 8 and 16 hr time points (Fig 7C). Caspase 8 inhibitor $cFLIP_L$ is processed in cells undergoing TNFR1-induced apoptosis (5). Interestingly, while no association of $cFLIP_L$ with caspase 8 was detected in untreated 293FLAG-XEDAR cells, a 43/41 kDa cleavage fragment of $cFLIP_L$ was found associated with caspase 8 in cells treated with EDA-A2 for 8 and 16 hr, respectively (Fig 7C). However, we failed to detect any full-length $cFLIP_L$ in the caspase 8 complex, suggesting that like the situation with Fas- and TNFR1-induced apoptosis, XEDAR-induced apoptosis is associated with complete processing of $cFLIP_L$. Remarkably, no recruitment of XEDAR or TRADD was detected to caspase 8-associated secondary complex at any time points (Fig 7C). Taken together, the above results suggest that, XEDAR-induced apoptosis is mediated via the formation of a secondary complex between caspase 8, caspase 10 and FADD.

DISCUSSION

In this report we demonstrate that XEDAR, which lacks a death domain, is capable of inducing apoptosis. In this context, XEDAR resembles the recently isolated *Drosophila* TNFR homolog Wengen, which also lacks a death domain but is capable of inducing apoptosis via a caspase-dependent mechanism (30,31). Wengen induced apoptosis is also dependent on the JNK pathway and can be inhibited by the JNK phosphatase Puckered (31). We have previously demonstrated that EDA-A2/XEDAR signaling can induce JNK activation (19) and it is conceivable that, akin to Wengen, JNK activation also contributes

to XEDAR-induced apoptosis. XEDAR may represent an early stage in the evolutionary history of the death receptors prior to the emergence of death domain, which probably appeared subsequently to hasten and augment the apoptotic process. Consistent with the above hypothesis, we have observed that EDA-A2 is a relatively weak and slow inducer of apoptosis as compared to TRAIL. Similarly, we and others have previously reported that Wengen and Eiger are relatively weak inducers of apoptosis in the *Drosophila* cells (31-33).

The evolutionary relationship between XEDAR and Wengen is also supported by structural similarities between the two receptors and their respective ligands. In addition to the lack of a death domain in their cytoplasmic domains, both XEDAR and Wengen share structural similarities in their NH2-terminal regions. Thus, XEDAR is a type III transmembrane protein and lacks an NH2-terminal signal peptide and differs in this aspect from the majority of mammalian TNF family receptors which are type I transmembrane proteins. Similarly, Wengen is believed to either lack an NH2-terminal signal peptide or to possess an atypical signal peptide (30,31). The structural similarity between XEDAR and Wengen also extends to their ligands, EDA-A2 and Eiger, respectively. Both Eiger and EDA are type II transmembrane proteins which differ from other mammalian TNF family ligands in possessing a relatively long extracellular domain that contains a unique juxtamembrane subdomain not seen in other ligands of this family. This domain is rich in aspartic acid residues in the case of Eiger and glycine residues in the case of EDA and has been postulated to help in ligand oligomerization (31-33).

A recent study reported that XEDAR-deficient mice were indistinguishable from their wild-type litter-mates (18). Although this study did not focus on the induction of apoptosis in XEDAR-null animals, its negative results may be explained by the fact that XEDAR plays a redundant role in the regulation of apoptosis during embryonic development. Consistent with the above hypothesis, we and others have previously reported that overexpression of EDAR and TAJ/Troy, two homologs of XEDAR, which are also highly expressed during embryogenesis, can induce cell death (20,21,34). Finally, it is possible that XEDAR-induces apoptosis only in limited organs/tissues and in specific cellular contexts and subtle defects in apoptosis induction in XEDAR-deficient animals might fail to yield an overt developmental phenotype.

Transgenic expression of soluble EDA-A2, but not EDA-A1, was recently reported to lead to birth of thin and listless animals which died within 1 month of birth (18). Histological analysis of affected animals revealed multifocal skeletal muscle degeneration, which was absent in EDA-A2 transgenic mice lacking XEDAR expression, thereby suggesting that it was a direct consequence of XEDAR signaling and not a non-specific effect of excessive EDA-A2 protein (18). Although the downstream events involved in EDA-A2-induced myodegeneration are unclear at the present, it is conceivable that caspase activation and induction of cell death plays a role in this process. EDA-A2-induced caspase activation and cell death may also play a role in tissue remodeling/differentiation in organs showing XEDAR expression in the adult life. Finally, EDA-A2-induced apoptosis may be exploited for the treatment of cancers showing XEDAR expression.

In this report, we present evidence that XEDAR utilizes a novel mechanism for activation of the caspase cascade and induction of apoptosis. Like the situation with TNFR1, activation of caspase 8 during XEDAR signaling is achieved via the formation of a secondary complex containing caspase 8, 10 and FADD, which does not contain the receptor. However, unlike the situation with TNFR1, the pro-apoptotic complex formed during XEDAR induced cell death lacks TRADD. Thus, our study suggests that TRADD is not essential for the formation of complex II containing caspase 8, 10 and FADD. In this context, it is important to point out that although TRADD has been postulated to play a role in TNFR1 induced apoptosis, genetic evidence to support its involvement in this process has been lacking so far. In the light of our results suggesting that XEDAR signaling can lead to the formation of complex II without TRADD and that siRNA-mediated silencing of TRADD expression failed to block TNFα-induced apoptosis, the role of TRADD in TNFR1-induced apoptosis may require re-examination.

We have observed a difference in the relative abilities of FADD-DN and FADD siRNAs to block EDA-A2 *vs* TRAIL-induced apoptosis. This discrepancy might be explained by the fact that while FADD is involved in caspase 8 activation during both EDA-A2- and TRAIL-induced apoptosis, it does so via two distinct complexes. Presumably, a greater amount of FADD is required for the formation of the DISC and subsequent caspase 8 activation during TRAIL signaling as compared to the formation of complex II during EDA-A2-induced apoptosis. It is also conceivable that while FADD may be absolutely required for DISC formation and subsequent caspase 8 activation via FasL and TRAIL, it

may facilitate the formation and/or activity of the caspase 8-containing secondary complex following EDA-A2 signaling without being absolutely essential for this process.

While our study does not reveal the nature of the trigger that could potentially lead to the formation of secondary complex capable of activating caspase 8 during XEDAR signaling, recent studies of TNFR1-induced apoptosis may provide some clues. It has been proposed that relative level of caspase regulator $cFLIP_L$ may control the activation of caspase 8 in complex II during TNFR1-induced apoptosis by controlling the access of caspase 10 to caspase 8 (5,9). In the current study, we demonstrate that caspase 8-associated $cFLIP_L$ is completely cleaved to its 43/41 kDa fragment during EDA-A2-induced apoptosis and its forced over-expression blocks EDA-A2-induced cell death. Thus, $cFLIP_L$ may be the final arbiter of caspase 8 activation during XEDAR signaling. Finally, in the case of TNFR1-signaling, it has been proposed that the dissociation of complex II from the receptor and its localization in the cytosol, or its association with the cytoskeleton may facilitate caspase recruitment and activation by potentially bringing the complex in proximity to apoptotic proteins (9). It is conceivable that a similar mechanism may be operative during XEDAR-induced apoptosis.

Acknowledgments

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Figure Legends

Figure 1: Induction of apoptosis by EDA-A2.

A. 293F-XEDAR-L cells were treated with control (PBS) or EDA-A2 (50 ng/ml). Twelve hours after treatment, cells were stained with YOPRO-1, which stains the nuclei of dead cells that have lost their plasma membrane integrity, and Hoechst 33342 dyes, respectively. Cells were photographed under phase-contrast and fluorescent microscopes. Inset shows the nuclear morphology of EDA-A2-treated cells as demonstrated by YOPRO-1 staining.

B. Hela-XEDAR-L cells were treated with actinomycin-D (200 ng/ml) alone or along with EDA-A2 (50 ng/ml). 12 hours after treatments, cells were stained with Hoechst-33342 and photographed under a fluorescent microscope.

C. XEDAR-Fc completely blocks EDA-A2 induced cell death while EDAR-Fc has no effect. 293F-XEDAR-L cells were treated with EDA-A2 alone or along with XEDAR-Fc or EDAR-Fc. 12 h after treatment, cells were stained with YOPRO-1 and Hoechst 33342 dyes, respectively and photographed under a fluorescent microscope.

D. Treatment with TNFα alone fails to induce apoptosis in 293F-XEDAR-L cells. 293F-XEDAR-L cells were treated with control (PBS), EDA-A2, TNFα (10ng/ml) or TNFα plus actinomycin D (200 ng/ml). Twelve hours after treatment, cells were stained with Hoechst 33342 and percent apoptotic cells were counted under a fluorescent microscope.

E-F Blockage of TNFα/TNFR1 signaling fails to block EDA-A2 induced apoptosis. 293F-XEDAR-L cells were treated with control (PBS), EDA-A2 or TNFα plus actinomycin D in the absence or presence of XEDAR-Fc or TNFRI-Fc (E) or a

neutralizing antibody against TNFα (F) and percent apoptotic cells counted as described for *1D*. Values shown are mean ±SE of a representative of two independent experiments performed in duplicate.

Figure 2: Transient transfection of XEDAR induces cell death.

A. 293T cells (1.5-3 x 10^5) were transfected with the indicated plasmids (500 ng) along with β-galactosidase- and GFP-encoding plasmids (75 ng each). 36 h post-transfection, cells were examined under a fluorescent microscope and photographed or were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as described previously (21). XEDAR- and TNFR1-transfected cells have a dark rounded appearance, condensed nuclei and are becoming detached from the plate, whereas cells transfected with the empty vector have normal morphology.

B. A schematic representation of XEDAR expression constructs. The XEDAR-L isoform differs from the XEDAR-s isoform by the presence of additional 21 amino acids (aa 173-192) in the juxtamembrane region of the cytoplasmic domain. N172 is a C-termial deletion mutant.

C. Induction of apoptosis by XEDAR-L and -s isoforms. 293T cells were transfected with the indicated plasmids along with pEGPF as described in Fig 2A. Apoptotic cells were counted 36 h after transfection on the basis of their morphology.

D. L-929 cells were transfected with the indicated plasmids along with an EGFP-encoding plasmid (75 ng) using Lipofectamine (Invitrogen) and examined under a fluorescent microscope.

Figure 3: Activation of caspase 8 and 3 during EDA-A2-induced apoptosis.

A. 293Flag-XEDAR cells were left untreated (control) or treated with EDA-A2 (50 ng/ml) or TRAIL (50 ng/ml). After the indicated time intervals, cell lysates were prepared and analyzed for cleavage of caspase 8, 3 and PARP by Western blotting. FL, Full length.

B-C. Caspase 3 and 8 activities were assayed in the cell extracts of 293Flag-XEDAR cells treated with EDA-A2 or TRAIL for 20 hours. Enzymatic activities were estimated as a function of time using a fluorometric assay to determine the turnover of peptide substrates DEVD-AFC (for caspase3) and IETD-AFC (for caspase8). The data shown is mean relative fluorescent units (RFU) per minute ±SE of duplicate assays and is representative of three independent experiments.

Figure 4. Activation of intrinsic caspase pathway during EDA-A2-induced apoptosis.

A. 293Flag-XEDAR cells were left untreated (control) or treated with EDA-A2 (50 ng/ml) or TRAIL (50 ng/ml). After the indicated time intervals, cell lysates were prepared and analyzed for cleavage of caspase Bid and caspase 9 by Western blotting. FL, Full length.

B. Caspase 9 activity was assayed in the cell extracts of 293Flag-XEDAR cells treated with EDA-A2 or TRAIL for 20 hours using LEHD-AFC as a substrate.

C. EDA-A2 induces release of cytochrome *c* and Smac form mitochondria. 293Flag-XEDAR cells were treated with EDA-A2 or TRAIL for 12 hours and then fractionated essentially as described (35) to separate the soluble cytosolic proteins

from the heavy membrane (HM) fraction, which contained the mitochondria. Western blot analysis was then performed with antibody against cytochrome C (BD Pharmingen) and Smac (Oncogene).

Figure 5: Caspase 8 activation is essential for EDA-A2-induced apoptosis.

A. 293Flag-XEDAR cells were treated with DMSO or 30 μM of the indicated caspase inhibitors for 30 minutes prior to treatment with EDA-A2. Twelve hours after treatment, cells were stained with Hoechst 33342 and counted for live and dead cells to determine percent apoptotic cells. Values shown are mean ± S.E. of a representative of two independent experiments performed in duplicate.

B. CrmA blocks EDA-A2 induced cell death. 293Flag-XEDAR cells were transfected with an empty vector or CrmA (500 ng) along with a pRcRSV/LacZ reporter plasmids. 12 hours after transfection, cells were treated with control or EDA-A2 for an additional 12 hours. Cells were fixed, stained and counted to determine percent apoptotic cells as described in Fig 1A. Values shown are mean±SE of a representative of two independent experiments performed in duplicate.

C. A dominant negative mutant of caspase 8 (C360S) and $cFLIP_L$/MRITα1 blocks EDA-A2 induced cell death. The experiment was performed essentially as described for 5B.

D-F. Knockdown of caspase 8 expression by siRNA blocks EDA-A2-induced cell death. 293Flag-XEDAR cells were transfected with siRNAs against lamin A/C (control) or caspase 8. 48 hours post-transfection, cells were treated with control (PBS), EDA-A2 or TRAIL for 12 hours. Hoechst 33342 stained cells were photographed (F) or counted to determine the percent apoptotic cell *(D)* as described in *(A)*. Cell lysates

were analyzed by Western blotting to show silencing of caspase 8 expression in representative samples *(E)*.

G&H. Downregulation of caspase 8 expression blocks caspase 3 activation by EDA-A2 in XEDAR cells. 293Flag-XEDAR cells were transfected with control (lamin A/C) or caspase 8 siRNAs. 48 hours post-transfection, cells were treated with EDA-A2 for the indicated time intervals. Cell lysates were analyzed by Western blotting for cleaved caspase 3, 8 and PARP, respectively *(G)*. Caspase 3 activity was assayed in the EDA-A2-treated samples (16 h time-point) using DEVD-AFC as a substrate *(H)*.

Fig 6. Role of FADD in EDA-A2-induced apoptosis.

A. DN-FADD protects cells form EDA-A2-induced cell death. 293Flag-XEDAR cells ($2x10^5$) were transfected with an empty vector or different amounts (250 ng and 500 ng) of DN-FADD expression plasmid along with a β-galactosidase reporter plasmid in duplicate in each well of a 24-well plate. The total amount of transfected DNA was kept constant by adding empty vector. Cells were fixed, stained, and percentage of apoptotic cells determined based on criteria described in Fig. 2A. Values shown are mean ±SE of a representative of three independent experiments performed in duplicate.

B. siRNA-mediated knock-down of FADD expression protects cells from EDA-A2 induced cell death. 293Flag-XEDAR cells were transfected with the indicated siRNA duplexes. 40-60 hours post-transfection cells, were treated with EDA-A2 or TRAIL. Apoptotic cells were determined based on Hoechst 33342 staining. Values shown are

mean ±SE of a representative of three independent experiments performed in duplicate.

C. Western blot analysis of cell lysate shows downregulation of endogenous FADD expression by respective siRNAs. Lamin siRNA was used as a control.

D. siRNA-mediated knock-down of TRADD expression fails to protect cells from EDA-A2 or TNFα (plus actinomycin D)-induced cell death. The experiment was performed essentially as described for 6B except actinomycin D (200ng/ml) was added to cells treated with TNFα. SiRNA against caspase 8 was used as a positive control.

E. Western blot analysis of cell lysate shows downregulation of endogenous TRADD and caspase 8 expression by respective siRNAs.

Fig 7. Lack of recruitment of endogenous TRADD, FADD or caspase 8 to XEDAR during EDA-A2 signaling.

A. 293Flag-XEDAR cells were treated with control (-) and EDA-A2 (+) for 10 minutes. Total cell lysates (CL) were immunoprecipitated with FLAG beads (M2) (Sigma). Endogenously expressed co-immunoprecipitated proteins were detected by Western blot with the indicated antibodies. TRAF3 and 6 are recruited to XEDAR in a ligand-dependent manner but FADD, TRADD caspase 8, RIP-1 or RICK/RIP2 fail to do so.

B. 293T cells were transfected with an empty vector, Flag-tagged XEDAR (F-XEDAR) or DR4 (F-DR4) plasmids. 24 hours after transfection, cells were lysed and total cell lysates (CL) were immunoprecipitated with FLAG beads (M2). Endogenous proteins that co-immunoprecipitated with FLAG-XEDAR and FLAG-DR4 were detected by Western blotting with the indicated antibodies.

C. Formation of a secondary complex containing caspase 8 during EDA-A2-induced apoptosis. 293Flag-XEDAR cells were treated with EDA-A2 for the indicated time intervals and immunoprecipitation performed using an anti-caspase 8 antibody. Endogenously expressed co-immunoprecipitated proteins were detected by Western blot with the indicated antibodies. Cleaved proteins are indicated with filled arrowheads while open arrowheads point to their full-length forms.

Fig.1

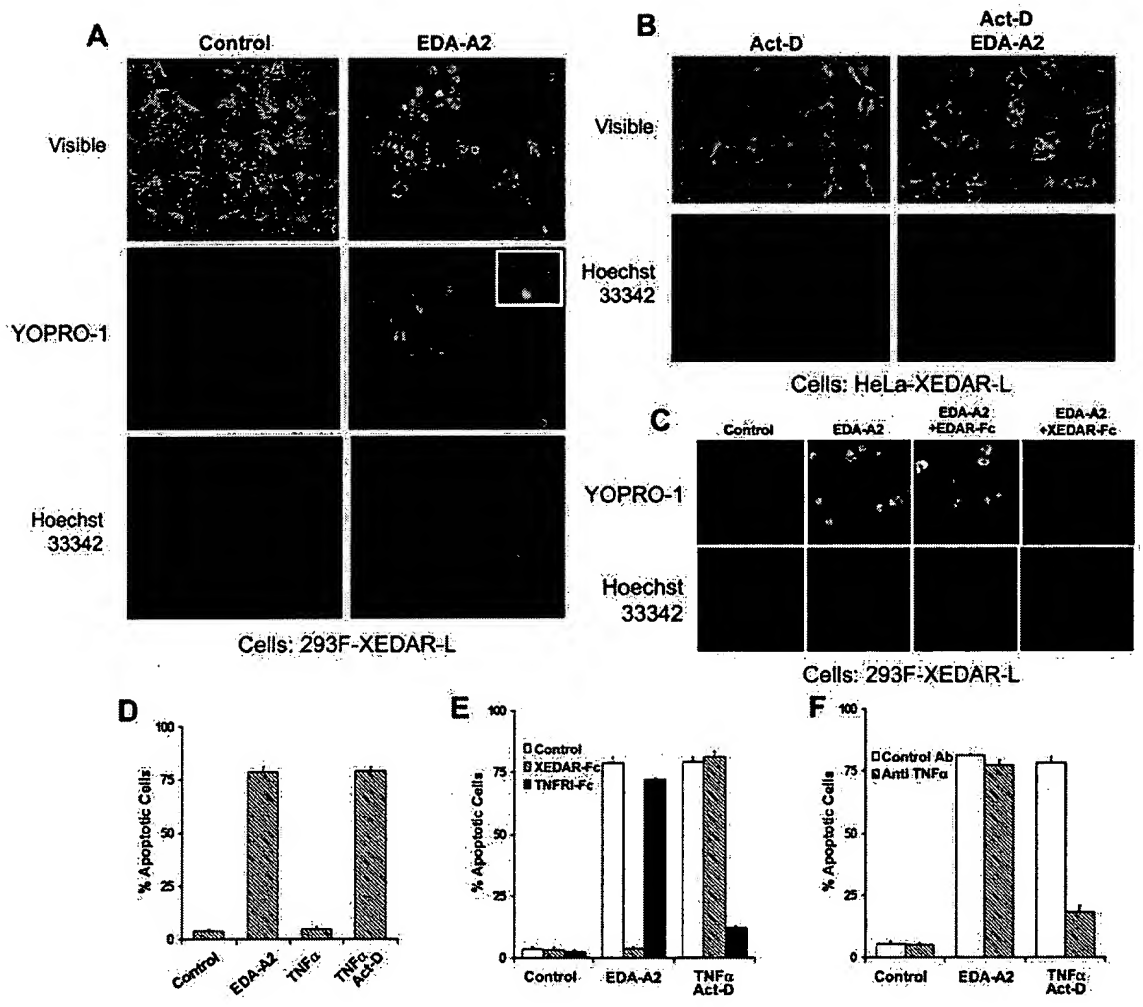
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Fig. 2

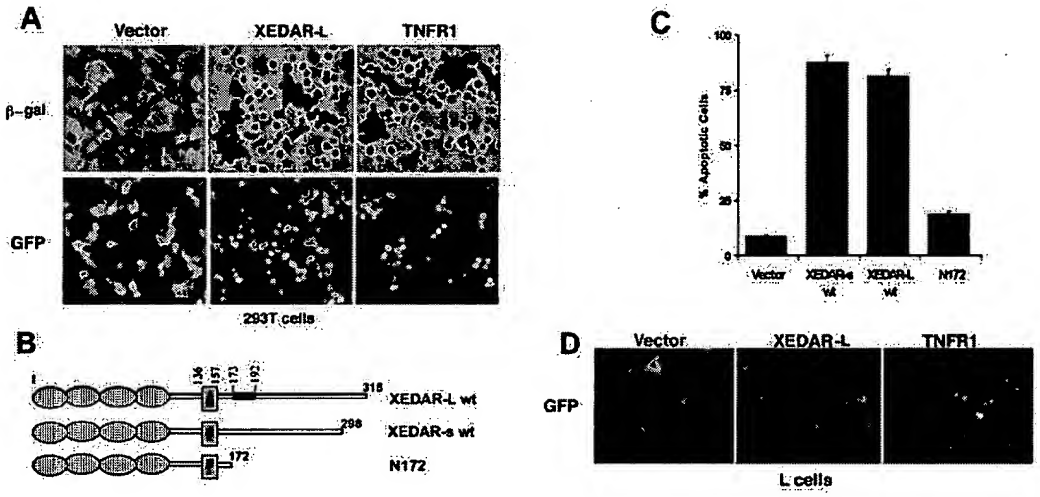
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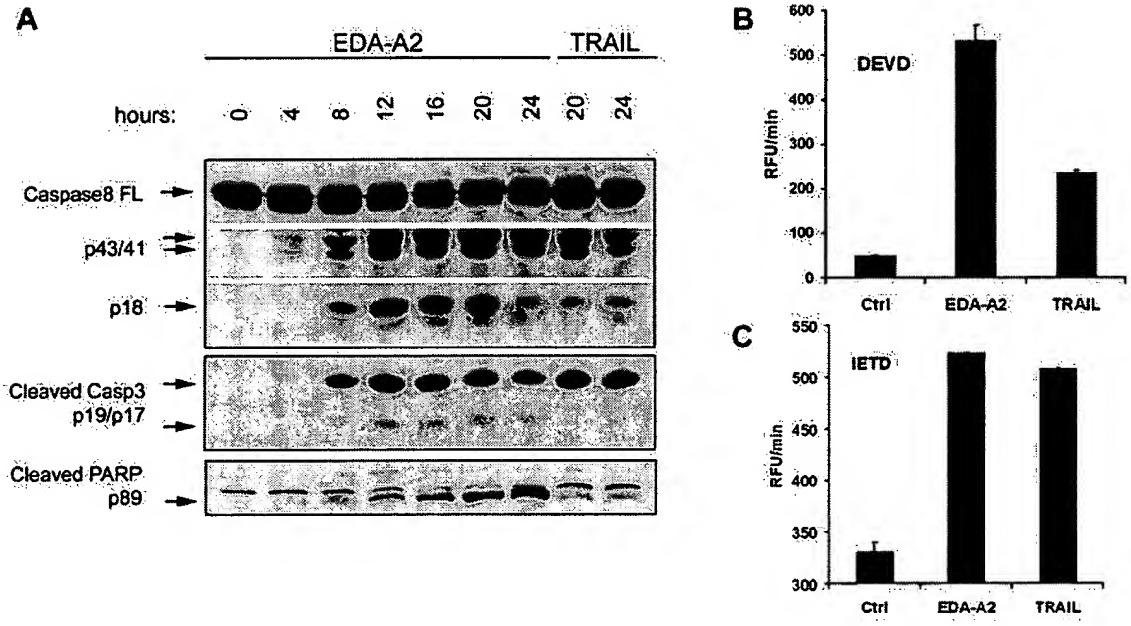
Fig. 3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TRAIL
C
IETD
RFU/min
550
500
450
400
350
300
Ctrl
EDA-A2
TRAIL

Fig. 4

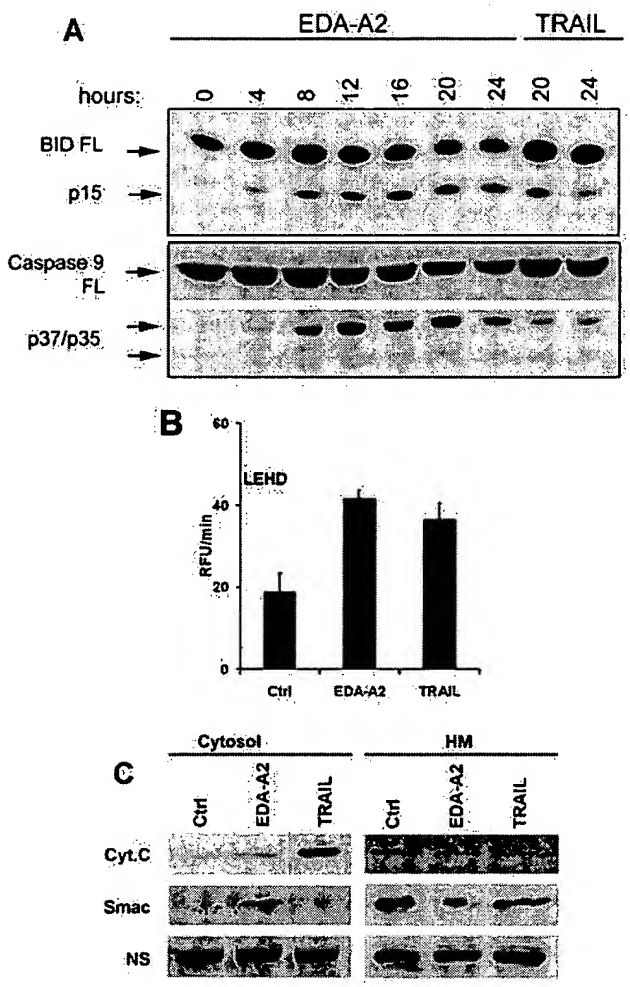

A
EDA-A2
TRAIL
hours:
0
4
8
12
16
20
24
20
24
BID FL
p15
Caspase 9 FL
p37/p35
B
LEHD
RFU/min
60
40
20
0
Ctrl
EDA-A2
TRAIL
C
Cytosol
HM
Ctrl
EDA-A2
TRAIL
Ctrl
EDA-A2
TRAIL
Cyt.C
Smac
NS

Fig. 5

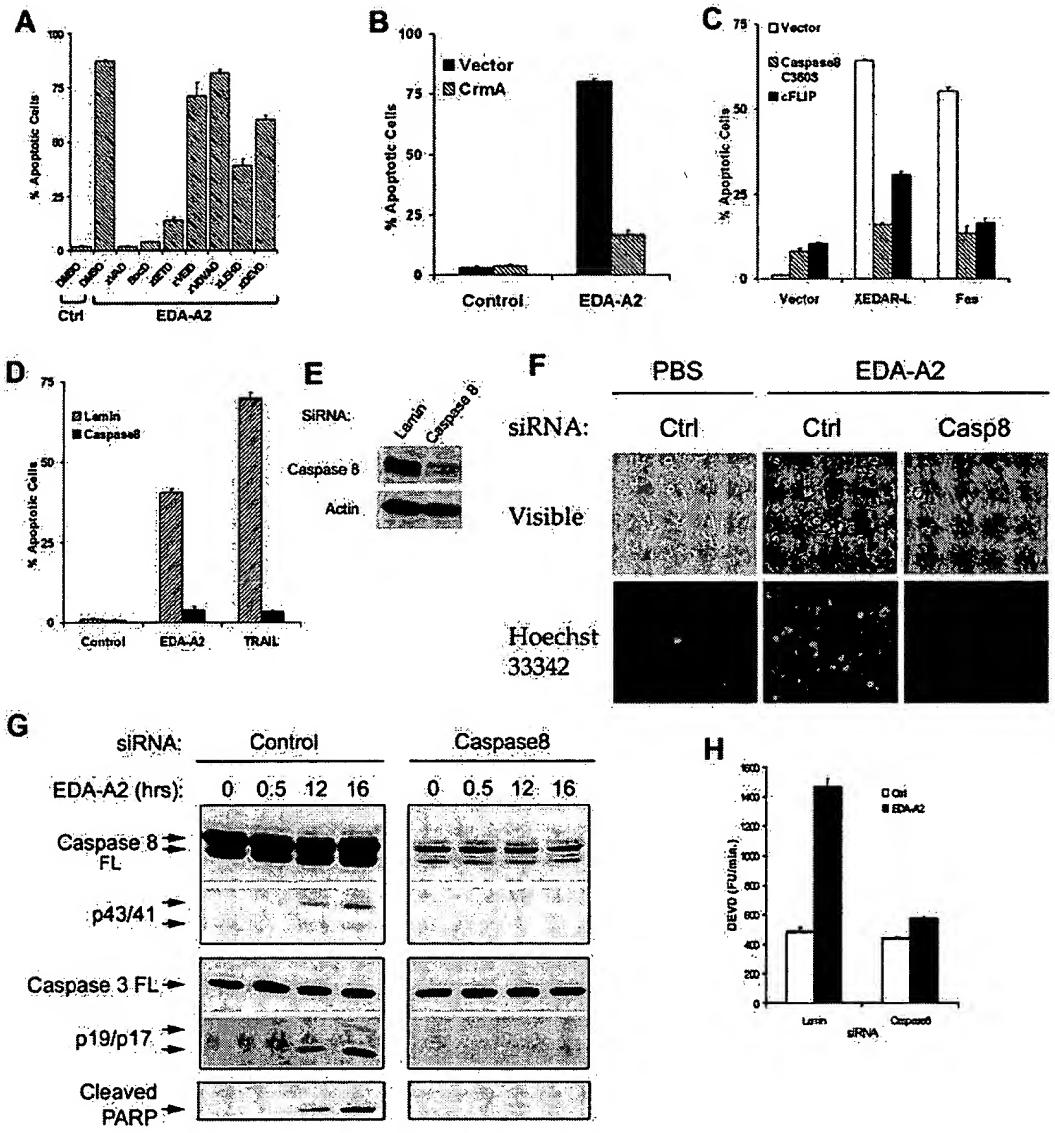

A
% Apoptotic Cells
DMSO
Ctrl
EDA-A2
B
Vector
CrmA
% Apoptotic Cells
Control
EDA-A2
C
Vector
Caspase8 C360S
cFLIP
% Apoptotic Cells
Vector
XEDAR-L
Fas
D
Lamin
Caspase8
% Apoptotic Cells
Control
EDA-A2
TRAIL
E
SiRNA:
Lamin
Caspase 8
Caspase 8
Actin
F
PBS
EDA-A2
siRNA:
Ctrl
Ctrl
Casp8
Visible
Hoechst 33342
G
siRNA:
Control
Caspase8
EDA-A2 (hrs):
0 0.5 12 16
0 0.5 12 16
Caspase 8 FL
p43/41
Caspase 3 FL
p19/p17
Cleaved PARP
H
Ctrl
EDA-A2
DEVD (FU/min.)
Lamin
Caspase8
siRNA

Fig. 6

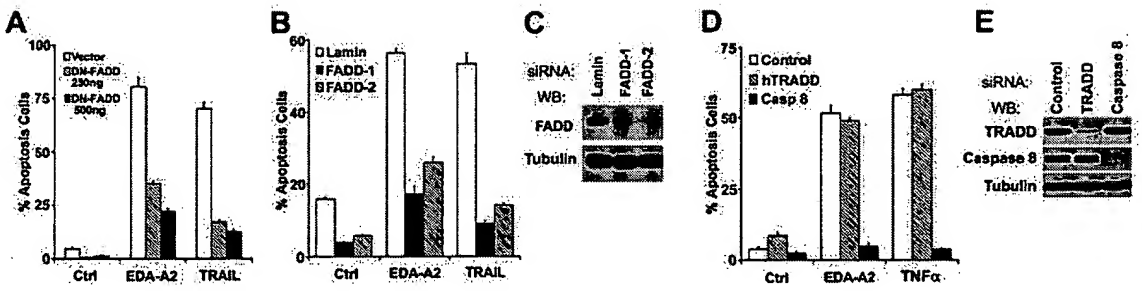
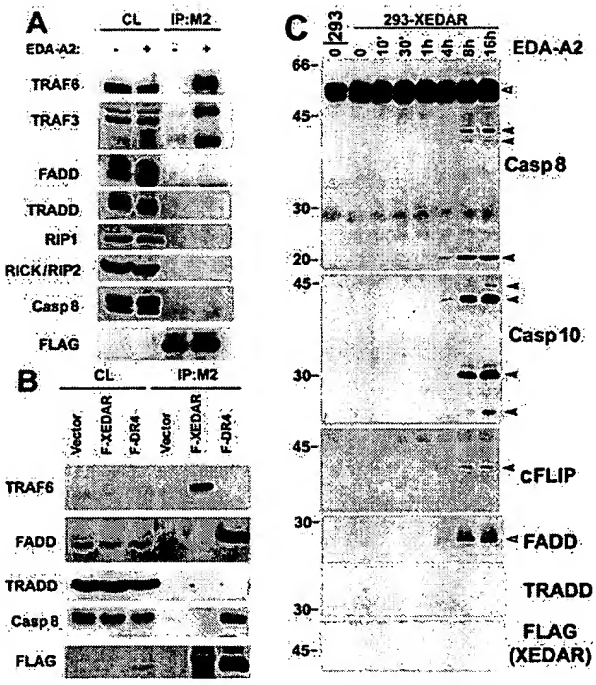

A
% Apoptosis Cells
Vector
EDN-FADD 250ng
EDN-FADD 500ng
Ctrl
EDA-A2
TRAIL
B
% Apoptosis Cells
Lamin
FADD-1
FADD-2
Ctrl
EDA-A2
TRAIL
C
siRNA:
Lamin
FADD-1
FADD-2
WB:
FADD
Tubulin
D
% Apoptosis Cells
Control
hTRADD
Casp 8
Ctrl
EDA-A2
TNFα
E
siRNA:
Control
TRADD
Caspase 8
WB:
TRADD
Caspase 8
Tubulin

Fig. 7


A
CL
IP:M2
EDA-A2:
TRAF6
TRAF3
FADD
TRADD
RIP1
RICK/RIP2
Casp8
FLAG
B
CL
IP:M2
Vector
F-XEDAR
F-DR4
TRAF6
FADD
TRADD
Casp8
FLAG
C
293
293-XEDAR
0
0
10'
30'
1h
4h
8h
16h
EDA-A2
66-
45-
Casp8
30-
20-
45-
Casp10
30-
45-
cFLIP
30-
FADD
TRADD
30-
FLAG
(XEDAR)
45-